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Zoological Research

Human Pro-insulin Transgenic Calf Derived from Somatic Cell Nuclear Transfer

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Abstract: The current study was undertaken to evaluate the possibility of producing a human pro-insulin transgenic cow by means of somatic cell nuclear transfer (SCNT). A double selection system, Neomycin resistance (Neo^r) gene and enhanced green fluorescent protein (EGFP) gene linked through an inner ribosomal entry site (IRES) sequence directed by a Cytomegalovirus (CMV) promoter, was used for enrichment and selection of the transgenic cells and preimplantation embryos. Transgenes were introduced into bovine fetal fibroblast cells (BFF) cultured *in vitro* through electroporation (900 V/cm, 5 ms). Transgenic bovine fibroblast cells (TBF) were enriched through addition of G418 in culture medium (800 μg/mL). Before being used as a nuclear donor, the TBF cells were either cultured in normal conditions (10% FBS) or treated with serum starvation (0.5% FBS for 2-4 days) followed by 10 hours recovery for G1 phase synchronization. Transgenic cloned embryos were produced through GFP-expressing cell selection and SCNT. The results were the percentage of blastocyst development following SCNT was lower using TBF than BFF cells (23.2% VS 35.2%, $P < 0.05$). No difference in the percentage of cloned blastocysts between the two groups of transgenic nuclear donor of normal and starvation cultures were observed (23.2% VS 18.9%, $P > 0.05$). Two to four GFP-expressing blastocysts were transferred into the uterus of each synchronised recipient. One pregnancy from of seven recipients (21 embryos) was confirmed by rectum palpation 60 days after embryo transfer and one recipient has given birth to a calf at term. PCR and DNA sequencing analysis confirmed that the calf was produced using human proinsulin transgenic animal.

Key words: Somatic cell nuclear transfer; Human pro-insulin; EGFP; Transgenic calf

利用体细胞核移植技术制作人胰岛素原转基因牛

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摘要: 通过体细胞核移植技术制作了人胰岛素原转基因牛。在 CMV 启动子指导下以内部核糖体进入位点序列(IRES)连接的新霉素抗性基因和绿色荧光蛋白基因组成了双重标记基因的筛选系统, 用于转基因细胞的富集以及细胞和植入前胚胎的筛选。转基因通过电穿孔的方法(900 V/cm, 5 ms)转入体外培养的牛胎儿成纤维细胞, 基因转染细胞在添加 G418(800 μg/mL)的培养基中培养 10 天以富集转基因细胞。选择表达绿色荧光蛋白的转基因细胞作为核供体进行体细胞核移植, 重构胚经体外培养至囊胚阶段, 选择表达绿色荧光蛋白的囊胚进行胚胎移植。为比较基因转染以及供体细胞所处周期对转基因细胞核移植胚胎发育的影响, 用作核移植供体的转基因细胞或非转基因细胞先饥饿培养 2—4 天(0.5% FBS), 然后恢复培养(10% FBS) 10 h 使细胞同步

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化于 G1 期，以正常培养的细胞作为对照进行核移植。结果表明，转基因细胞作为核供体得到的核移植胚胎的体外囊胚发育率低于以非转基因细胞为核供体的对照组（23.2% VS 35.2%，$P<0.05$）；转基因细胞周期同步化处理与否对其克隆胚囊胚发育率无显著影响（23.2% VS 18.9%，$P>0.05$）。胚胎移植后 2 个月直肠检查发现 7 头受体牛（每头移植 2—4 枚胚胎）中有一头妊娠，并最终发育足月产下一头小牛。聚合酶链反应（PCR）检测和 DNA 测序分析表明其为转人胰岛素原基因的转基因克隆牛。

关键词：体细胞核移植；人胰岛素原；绿色荧光蛋白；转基因牛
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Large scale production of human recombinant proteins in transgenic animals is one of the major successes of biotechnology. The mammary glands of large animals is well suited for the production of these proteins (Louis, 2000). Therapeutic proteins, such as alpha-1 antitrypsin, antithrombin, and several monoclonal antibodies, have been produced in the milk of transgenic animals (Wright et al, 1991; Edmunds et al, 1998; Pollock et al, 1999). The benefits of this technology include high production yields, low capital investment, and the elimination of reliance on products derived from human blood. Until recently, the only reliable method available for producing transgenic farm animals has been pronuclear microinjection. However, only 0.5% -3% of microinjected embryos gave rise to transgenic offspring (Ebert et al, 1994; Behboodi et al, 2001). The emerging use of transfected cultured somatic cells as nuclear donors for nuclear transfer (NT) has several advantages over microinjection, and has facilitated the generation of transgenic animals, especially in domestic species (Schnieke et al, 1997; Baguisi et al, 1999; Onishi et al, 2000). This method allows the selection of transgenic cells in a relatively long period, *in vitro*, by using drug selection marker genes usually contained in transgene constructs and even allows the selection of homologous recombinant cell clones, which results in gene targeting in domestic animals.

All animals created via NT from such selected cells should be but are not always transgenic. Several studies have observed a so-called bystander effect, where transgenic cells, which express the antibiotic-resistance gene (such as Neo^r), provide the protection to nearby non-transgenic cells either by secretion of the gene product into the medium or by direct cell-to-cell contact (Bondioli et al, 2001; Echelard et al, 2002). As a result, many transfected colonies are mixed and contain both transgenic and non-transgenic cells. NT blastocysts derived from these donor cells were also mixed resulting in embryo transfer of non-transgenic embryos to recipients. Green fluorescent protein is a genetic reporter system derived from a bio-iluminescent jellyfish and it has been used successfully as a marker in DNA microinjection embryos (Takada et al, 1997; Keiser et al, 2001).

In this study, in order to produce transgenic cows expressing human insulin in milk, we employed both the Neo^r and EGFP genes to construct a double selection vector for the enrichment and selection of the transgenic nuclear donor cells and for a pre-screening of the preimplantation embryos. *In vitro* developmental capacities of NT embryos using transgenic fetal fibroblast cells versus the same cell line non-transfected and the influence of donor cell synchronization on transgenic cloned embryo development were also investigated.

1 Materials and Methods

1.1 Construction of pNEI vector

The pNEI vector using pGEM® -7Zf (+) as backbone contains two expression unit. The marker gene expression unite contains 0.58 kb of Cytomegalovirus (CMV) promoter, 0.97 kb of neomycin resistance (Neo^r) gene, 0.58 kb of inner ribosomal entry site (IRES) sequence, 0.72 kb of enhanced green fluorescent protein (EGFP) gene and a SV40 polyA. The human proinsulin gene expression unit contains 0.84 kb of bovine α-lactoalbumin promoter (pBLA), 1.34 kb of human pro-insulin genomic sequence (HI) including polyA signal and 3′ flanking region. To prepare the fragment for transfection, pNEI was linearized through digestion with *XhoI* then purified (Fig. 1).

1.2 Isolation, culture, and transfection of fibroblasts

Bovine fetal fibroblast (BFF) cells were isolated from a 3-month fetus as previously described (Yang et al, 2006). Briefly, samples were finely minced and tissues were plated in a 60 mm culture dish with DMEM/F12 medium (Gibco BRL, USA) supplemented with 10% fetal bovine serum (FBS) (TBD, Tianjin, P. R. C), 2 mmol/L L-glutamine (Sigma, USA) and 50 IU/mL (50 μg/mL) penicillin-streptomycin (Sigma, USA) and cultured in a humidified 5% CO_2 incubator at 37℃. 10^6BFF passage 2 cells were electroporated

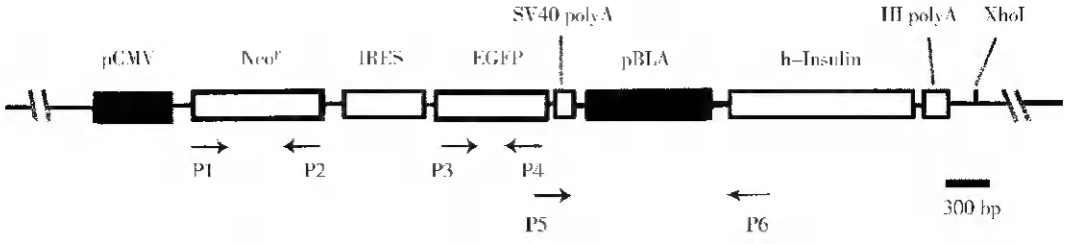

Fig. 1 Schematic representation of the pNEI transgene.

The marker gene expression cassette containing a 0.9 kb sequence of neomycin resistance gene (Neo^r) linked with a 0.7 kb enhanced green fluorescent protein gene (EGFP) was directed by a human cytomegalovirus immediate early promoter (pCMV) and ended with a SV40 polyA; A 1.34 kb fragment of human proinsulin gene genomic sequence including its polyA tail (HI polyA) was directed by a bovine α-lactoalbumin promoter (pBLA, 0.84 kb). The vector was linearized by XhoI digestion before transfection. The arrows indicate three pairs of PCR primer to detect the integration of the transgene.

(Gene Pulser Xcell™, BioRad, USA) with 20 μg/mL linerized pNEI vector with a single pulse of 180 V for 5 ms using a 2 mm Gap cuvette. Cells were then seeded into 6-well plates. G418 selection (800 μg/mL) was applied 48 h after seeding. After two weeks, selected colonies were pooled for fluorescence analysis and cryopreservation.

1.3 *In vitro* maturation of bovine oocytes

Bovine ovaries collected from a local slaughter house were transported to the laboratory in a 0.9% (V/V) NaCl solution and maintained at 25–30℃ until recovery of cumulus oocytes complexes (COCs). The follicular fluid with COCs was aspirated from 2–8 mm follicles through an 18-gauge needle connected to a syringe and recovered into a 100 mm culture dish. Only oocytes enclosed in compact cumulus with evenly granulated cytoplasm were selected for maturation. The COCs were washed three times in maturation medium, which consisted of bicarbonate-buffered TCM-199 (Gibco BRL, USA) supplemented with 10% (V/V) FBS, 0.005 IU/mL FSH (Antrin, Japan) and 1 mg/mL 17β-estradiol (Sigma, USA). A group of 100–300 COCs were cultured for maturation in a 30 mm culture dish containing 2 – 3 mL of maturation medium at 38.5℃ in a humidified atmosphere of 5% CO_2 for 18 hrs.

1.4 Nuclear transfer

The *in vitro* matured oocytes were denuded at 18 h post maturation (hpm) by pipetting in PBS-containing 0.1% hyaluronidase (Sigma, USA). The metaphase II-associated chromosomes were removed from oocytes at 18–22 hpm in 25 mmol/L Hepes buffered TCM199 medium containing 10% FBS, 7.5 μg/mL cytochalasin B (Sigma, USA) and 5 μg/mL Hoechst 33342 (Sigma, USA) by gentle aspiration of the polar body and the metaphase plate in a small amount of cytoplasm using a glass pipette 20 μm in diameter. The oocytes were exposed to ultraviolet light for 1–2 s to ensure the removal of the oocyte chromatin before and after the process. In the groups using transgenic cells as nuclear donors, only cells expressing GFP were picked for nuclear transfer. Non-transgenic cells were used as a control. A single donor cell was placed under the zona pellucida of the enucleated oocyte. The cell-cytoplast complexes were then fused by application of two DC pulses (1.8 kV/cm) for 20 μs with an interval of 1 sec using the Voltain EP1 Cell fusion system (Cryologic Inc., Australia). The fusion medium was 280 mmol/L manitol with 0.1 mmol/L $CaCl_2$, 0.1 mmol/L $MgCl_2$ and 0.01% PVA. The fusion rate was checked 30 min after the electrical pulse. The reconstructed embryos (fused) were activated through incubation in SOF medium containing 7% ethanol for 7 min then in SOF medium containing 2 mmol/L 6-DMAP (Sigma, USA) for 4 hrs. No more than 50 of the activated reconstructed embryos were settled in a well of a 4-well dish containing 500 μL SOF medium (with essential and non-essential amino acids) at 38.5℃ in 5% CO_2, 5% O_2 and 90% N_2, covered with mineral oil and were cultured for 7 days.

1.5 Selection of transgenic embryos

The blastocysts derived from SCNT were detected for expression of GFP under a fluorescent microscope to select the transgenic embryos 7 days after *in vitro* culture.

1.6 Embryo transfer

The herds of pure Holstein dairy cows used for this study as recipient cows were maintained under good agricultural practice at the farm of HuaQi, Inc. Day 7 (day 0 = fusion day) GFP positive blastocysts

were non-surgically transferred to synchronized recipients on day 7 of their estrus cycle (day 0 = estrus). Immediately prior to transfer to recipients, embryos were placed in equilibrated SOF medium, and transported to farm within 3 hours. Two-four embryos were transferred into the uterine horn of each recipient. Pregnancy was determined by rectum palpation starting on day 60 after embryo transfer.

1.7 Detection transgene of the cloned calf

In order to detect the transgene integration of the cloned calf, genomic DNA was extracted and PCR amplification was performed using three pairs of primers: one primer pair (p1 and p2) specific to Neo^r gene with expected product of 976 bp; one primer pair (p3 and p4) specific to EGFP gene with expected product of 658 bp and the last one (p5 and p6) specific to the sequence spanning human insulin expression unit and EGFP unit with expected product of 1160 bp.

P1 5′-GGAGCTAGCCGCATGATTGAACAAG-3′,
P2 5′-GTAGGATCCGAACTCCAGCATGAGA-3′
P3 5′-TGAAGGATGCCCAGAAGGTA-3′,
P4 5′-AGTTCACCTTGATGCCGTTC-3′
P5 5′-GACCACTACCAGCAGAACAC-3′,
P6 5′-CCAGGAAGAGGATGACAAGA-3′

Genomic DNA (300 ng) was amplified in a 50 μL PCR reaction containing 2.5 units Taq polymerase (Takara, Dalian, China) and its buffer, 1.5 mmol/L $MgCl_2$, 2 mmol/L dNTP, and 50 pmol specific primers. PCR amplification was carried out for 35 cycles with denaturing for 1 min at 94℃, annealing for 1 min at 58℃, extension for 40 sec at 72℃, and a final extension for 10 min at 72℃. Ten microliters of PCR products were loaded on a 1% agarose gel for electrophoresis and stained with ethidium bromide. The PCR products isolated from gels were cloned into a T-vector using a TA Cloning Kit (Tiangen Biotech, China) and were sequenced (Takara, Dalian, China).

1.8 Experimental designs

In order to investigate the effect of transfection of foreign DNA fragments on the development competence of SCNT embryos, transgenic or non-transgenic fibroblast cells cultured in normal conditions were used as donor cells. To determine the effect of the G1 stage synchronization method on transgenic cloning efficiency, transgenic fibroblast cells were serum starved (0.5% FBS) for 2–4 days followed by 10 hrs recovery (10% FBS) and then used as a nuclear donor. The percentage of cloned blastocysts derived from the three donor cell groups were compared.

In order to produce transgenic cloned calves, Day 7 GFP-expressing SCNT blastocysts were transferred into the uterine horn of recipient cows on Day 7 after estrus.

1.9 Statistical analysis

All the data were analyzed by χ^2-test. A value of $P < 0.05$ was considered to be significantly different.

2 Results

2.1 Establishment of transgenic donor cells

The data was published previously (Yang et al, 2006). Briefly, transgenic BFF cells were selected out through the addition of G418 in medium for two weeks, and the transgenic cell clones were pooled and cryopreserved. The integration of Human pro-insulin transgene was confirmed through PCR detection.

2.2 Influences of donor cell treatment on NT embryo development

As shown in Tab. 1, the development to blastocyst was higher when embryos were reconstructed with the non-transfected cells than with the transfected ($P < 0.05$). Also as shown in Tab. 1, no significant differences in rate of blastocysts were observed between the two groups of transgenic cells as nuclear donor.

Tab. 1 Effects of donor cells on NT blastocysts development

Donor cell type		Oocytes	Doublets fused (%)	Blastocysts (%)
Transgenic	SSR	283	159 (56.2)[a]	30 (18.9)[b]
	NC	544	301 (55.3)[a]	70 (23.2)[b]
Non-transgenic		385	228 (59.1)[a]	77 (35.2)[b]

Different letters (b and c) in the same column indicate significantly different ($P < 0.05$). SSR: serum starvation and recovery, NC: normal culture.

2.3 GFP expression of NT blastocysts

Among the 100 cloned blastocysts derived from transgenic donor cells, GFP was detected under fluorescent microscope in 63 embryos (Fig. 2).

2.4 ET results and identification of human pro-insulin transgenic cow

In order to produce transgenic cloned cows, 21 GFP-expressing blastocysts were transferred into seven recipients (2–4 embryos/recipient). At Day 60 after embryo transfer, one pregnancy was confirmed by rectal palpation. Fortunately, the only pregnancy was carried to term and one calf was delivered (Fig. 3). Analysis of genomic DNA by PCR amplification revealed the integration of all three foreign genes (human insulin gene and two marker genes) in this calf, while

no transgene integration was detected in the surrogate female Holstein cows (Fig. 4). The PCR products were gel purified, cloned and sequenced. Sequence analysis confirmed the presence of transgenes in the transgenic cloned calf.

3 Discussion

Bondioli et al (2001) reported that 100% (2/2) of cloned pigs born from drug-selected cells were non-transgenic. In addition, Echelard et al (2002) reported that 4/6 (66.7%) cloned calves born from drug-selected CL53 cells were non-transgenic. In this study, a double selection system using EGFP Neo^r for overcoming the so called bystander effect was utilized successfully in bovine transgenic nuclear transfer. The calf developed to term was transgenic due to the application of the double selection system, which indicated that transgenic selection by Neo^r plus EGFP maybe a hopeful solution to the bystander problem in transgenic NT.

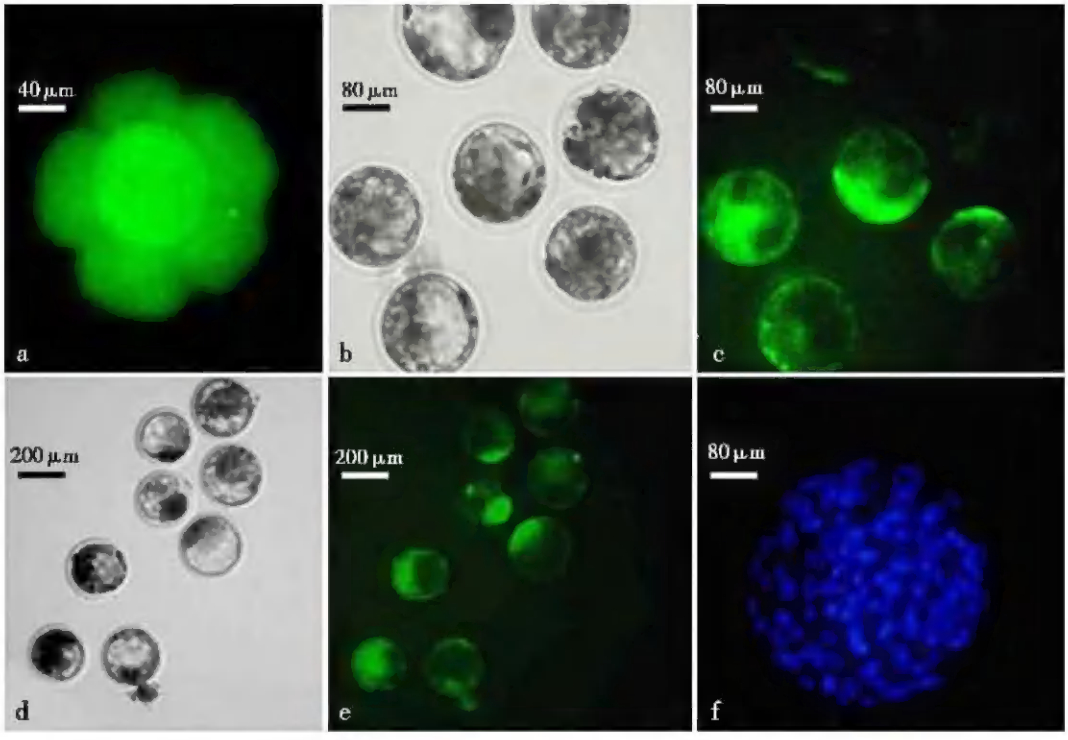

Fig. 2 Transgenic embryos produced by transgenic cloning

a, Detection of GFP in a 8-cell embryo under fluorescent microscope. b, Blastocysts under normal light microscope as control of c. c, Blastocysts under fluorescent microscope show a few embryos in which GFP were undetectable. d, Blastocysts under normal light microscope as control of e. e, Blastocysts under fluorescent microscope. f, Transgenic cloned blastocyst under UV pre-stained with Hochest33342 show the nuclears.

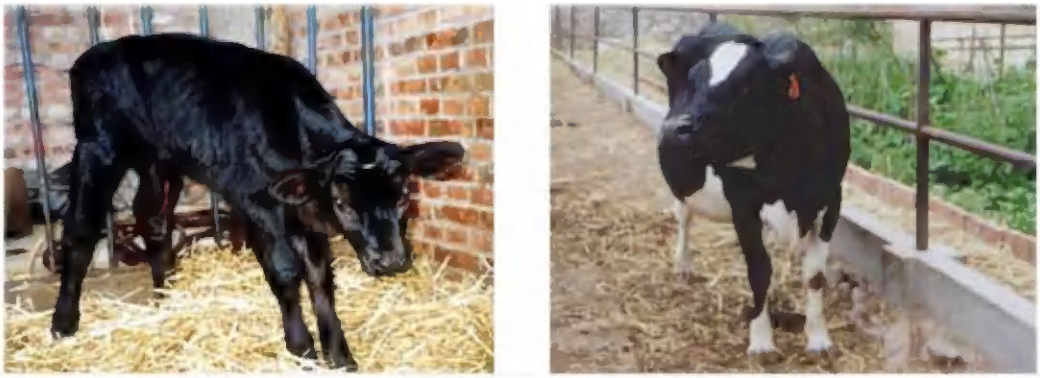

Fig. 3 The transgenic cloned calf named MengKe Ⅲ (left) and its surrogate mother (right, 1 month before delivery).

Born in 15th, Aug, 2005 in Inner Mongolia, China. Birth weight 55 kg, Delivered by Caesarean operation at day 280 after embryo transfer.

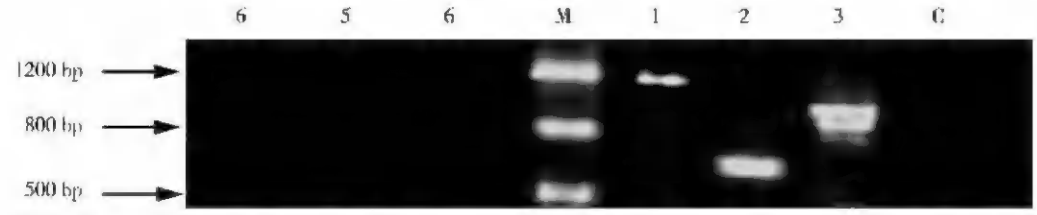

Fig. 4 Identification of Neo^r, EGFP and pBLA-HI integration in the cloned calf by PCR analysis of total DNA. Lanes 1-3: PCR products using DNA of the cloned calf as template. The primer pairs used in lane 1, 2 and 3 are specific for Neo^r, EGFP and EGFP + pLA-HI, respecfively. Lanes 4-6 were controls for ane 1-3 with DNA from surrogate female as template. Lane C is a control without template. M: DNA marker.

Since all transgenic donor cells were EGFP positive as detected when used as NT donor cells, it's unexpected that 37% of NT blastocysts derived from these cells appeared negative in our results. In addition, the somatic cell derived from the cloned calf can grow in media containing more than 1 000 μg/mL G418 for over two weeks, but GFP expression can't be observed (data not present). A similar result was observed by Chen et al (2002) and Gong et al (2004). The former explained that the observation was limited by the transient expression of GFP in some fibroblasts, gene silencing in the embryo or expression of GFP beyond our visual detection. The CMV promoter is well known to be prone to DNA methylation and is frequently silenced in the transgenic animals. That may be the reason for the 37% of the blastocysts in which the GFP was not detected in this study. Presumably, this phenomenon indicated that the transgene could be inserted into a site that is vulnerable to DNA methylation in these transgenic cloned blastocysts. To some extent, GFP selection at blastocyst stage in this study excluded some of these embryos to be transferred. Presumably, transgenes would be less influenced by the so called "site-effect" in the genome of transgenic offspring.

As indicated in Tab. 1, more NT embryos derived from non-transfected donor cells developed into blastocyst stage compared with that from transfected donor cells. However, examples of contradictory results have been observed in bovine. Some reports (Zakhartchenko et al, 2001; Bhuiyan et al, 2004) indicated a significant decrease in blastocyst rate when using transfected cells compared to non-transfected donor cells, while others reported no difference in blastocyst development rates between NT of transfected and non-transfected cells (Brophy et al, 2003). Zakhartchenko (Zakhartchenko et al, 2001) attributed their decreased blastocyst rate of embryos reconstructed with transfected fetal fibroblasts compared to non-transfected cells to the extended culture period required for transfection and selection processes, but not to the transgene *per se*. However, by using transfected and non-transfected adult bovine granulosa donor cells, Arat et al (2001) have demonstrated that cells cultured *in vitro* for a long period (15 passages) had better cleavage and blastocyst rates than cells from earlier passages (10, 11 and 13 passages). Another report (Bhuiyan et al, 2004) in the bovine SCNT demonstrated that transfected ear fibroblasts were less able to develop to the blastocyst stage than their non-transfected counterparts, regardless of passage number (early or late). It is more likely that discrepancies among the results reported up to now are due to the differences in vector type, transfection protocols (Bhuiyan et al, 2004), NT methods and donor cell culture conditions (Wells et al, 2003). Furthermore, the site of gene(s) integration(s), the transgene used and its possible interference in endogenous gene expression could influence results (Hodges and Stice, 2003). It has been reported that each colony resulting from transfection and selection in G418 has widely different development potential when used for NT (Dai et al, 2002). To reduce the chance of working with a colony with low developmental potential, Chen et al (2002) pooled the colonies after G418 selection. They pointed out that the use of early passage pools, compared with single colonies exposed to long-term culture during selection, has the added benefit of providing potentially healthier donor cells for NT. Compared with the use of individual colonies, this strategy increases the number of possible integration events used to produce offspring, thus increasing the probability of obtaining one or more highly expressing lines. The lower efficiency of NT with transgenic cells may be due to the processes of transfection and selection but may also be due to sampling errors related to the use of a single transgenic colony. In this study, in order to reduce the risk of long term selection culture in G418 medium and the

risk of mono-colony, we pooled the transgenic cells after G418 selection. However, the results still show a decrease in blastocyst development in transgenic cloning compared with that of non-transgenic cloning.

To date, investigations of the cell cycle (G0, G1, S and G2) for nuclear transfer have focused on the basic question concerning the DNA content of the donor cell to maintain correct ploidy after nuclear transfer and to avoid occurrence of chromatin abnormalities (Campbell et al, 1996). A number of studies have reported that donor cells, which are assumed to be at quiescence (G0), or at G1 phase, give better results in nuclear transfer (NT) than cells at other phases of the cell cycle (Collas et al, 1992). Whether G0 or G1 cells function better as donor cells is yet to be determined by detailed studies. While some researchers reported that serum starved (G0) cells support better embryo development (Wilmut et al, 1997; Cho et al, 2002), others reported that there were no differences in embryo development between G0 and G1 derived NT embryos (Cibelli et al, 1998; Korfiatis et al, 2001). However, Kasinathan et al (2001) reported that there was a higher number of viable offspring in the production when G1 cells were used as donor cells. Wells et al (2003) reported that there were more viable bovine NT animal production with non-transgenic cells at the G0 stage, and transgenic cells at the G1 stage of the cell cycle. A synchronization protocol which resulted in a cell population with a majority of donor cells in the G1 phase of the cell cycle was described in goat fibroblast cells, in which cells were serum starved (0.5% serum) for four days then recovery (normal culture) for 10 hours (Memili et al, 2004). In this study, effect of the synchronization treatment (the starvation then recovery treatment) of donor transgenic cells on the *in vitro* development of bovine transgenic NT embryos was evaluated. There were no significant differences in fusion rate and in blastocyst development between the two NT groups whether using donor transgenic cells pretreated or not using the G1 phase synchronization method. Thus there were no significant influence of G1 synchronization by serum starvation and stimulation on *in vitro* development of bovine transgenic NT embryos. No conclusion can be drawn about its influence on subsequent *in vivo* development due to the small number of embryos transferred in this study.

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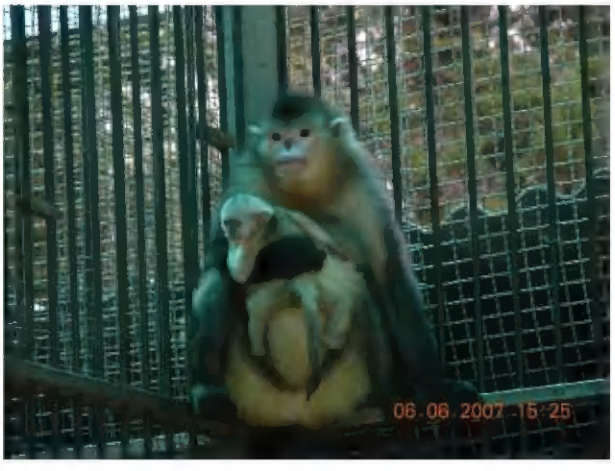
昆明动物研究所成功繁育我国第一头子二代滇金丝猴



滇金丝猴仅分布于云南西北部和藏东南高海拔地区，是我国一级保护动物，被 IUCN 列为最濒危的物种之一。

昆明动物研究所自 80 年代起采用多学科和现代生物学研究技术，对滇金丝猴的资源状况、区系分类、系统演化、遗传进化、繁殖生物学、行为生态学、分子遗传进化和保护生物学等方面进行了系统深入的综合研究。并首次引进滇金丝猴的种源进行人工饲养，开展迁地保护研究，现人工养殖的种群数已达 12 头，并成功建立了一整套驯养、管理、繁殖和疾病防治的科学方法体系。

2007 年 5 月 25 日，该种群第一头子二代滇金丝猴顺利出生，目前，健康状况良好。这是昆明动物研究所繁殖成功的第一头子二代滇金丝猴，也是我国人工繁殖成功的第一头滇金丝猴子二代动物。